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Inhibition of polyphenol oxidase and peroxidase activities on fresh-cut apple by simultaneous treatment of ultrasound and ascorbic acid

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ABSTRACT

The effects of ultrasound and ascorbic acid on activity changes of polyphenol oxidase and peroxidase, of fresh-cut apple during storage, were investigated. The combined treatment of ultrasound and ascorbic acid inactivated monophenolase, diphenolase, and peroxidase, whilst the individual treatment of ultrasound or ascorbic acid had inverse and limited inhibitory effect on the enzymes. The main protein bands had a molecular weight of approximately 63 kDa. A diffuse band, lacking the electrophoretic mobility of proteins, was observed after combined treatment. This investigation revealed that simultaneous treatment with ultrasound and ascorbic acid had synergistic inhibitory effects on several enzymes related to enzymatic browning.

1. Introduction

In recent years, a rapid market growth for fresh-cut fruits and vegetables has been observed due to the consumers' increased demand for convenience, fresh-like quality, and high nutritive value (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). Fresh-cut apples, in particular, are desired as a convenient snack for catering services to salad-bars, schools, and company cafeterias (Saftner, Abbott, Bhagwat, & Vinyard, 2005). Surface colour is one of the most important quality attributes because consumers usually judge the quality of fresh-cut fruits and vegetables on the basis of appearance. However, the shelf-life of fresh-cut products is restricted by physiological injury resulting from essential processing operations, including peeling, coring, and cutting. These processes cause browning on the cut surface which limits the development and commercialisation of fresh-cut fruits and vegetables. Therefore, the food industry is constantly searching for effective and safe means to control such problems (Kader, 2002; Sapers, Hicks, & Miller, 2002).

Polyphenol oxidase (PPO) and peroxidase (POD) are the enzymes involved in the browning process. Browning occurs almost instantly when the cell structure is destroyed, and the enzyme and substrate are mixed. PPO catalyses the hydroxylation of monophenols (monophenolase) and oxidation of *o*-diphenols to *o*-quinones (diphenolase), which subsequently polymerise to yield undesirable brown pigments in the presence of oxygen (Espín, García-Ruiz, Tudela, Varón, & García-Cánovas, 1998).

POD, an indicator of quality deterioration such as flavour loss and various biodegradation reactions, is also relevant to enzymatic browning since diphenols may function as reducing substrate in the enzyme reaction and could promote darkening in fruit and vegetable products during processing and preservation. Although POD is limited by the availability of electron acceptor compounds like hydrogen peroxide, its involvement in browning of various fruits and vegetables has been reported (Chisari, Barbagallo, & Spagna, 2007; Mdluli, 2005; Richard-Forget & Gauillard, 1997; Valderrama & Clemente, 2004).

Several studies have focused on the inhibition of enzymatic browning by ascorbic acid and thermal treatments. Ascorbic acid can reduce *o*-quinones, produced by PPO-catalysed oxidation of polyphenols, back to dihydroxy polyphenols and has been widely used as an antibrowning agent for processing of fruits and vegetables. However, the effect of ascorbic acid is temporary since once it is added, it is completely oxidised and *o*-quinones could accumulate, leading to browning pigment formation (Özoglu & Bayindirli, 2002; Rojas-Graü, Sobrino-López, Tapia, & Martín-Belloso, 2006). Therefore, ascorbic acid is insufficient in controlling browning and maintaining the commercial value of fresh-cut products. On the other hand, though thermal treatment effectively inactivates enzymes, non-thermal treatment is needed as a substitute to keep the fresh-like colour and texture of fresh-cut fruits and vegetables (Rico et al., 2007). Ultrasound causes enzyme inactivation by cell lysis using vibration energy, which produces cavitation bubbles and temporarily generates spots of extremely high pressure and

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temperature when imploded (Morris, Brody, & Wicker, 2007). Ultrasonication has been found to be more effective in inhibiting enzyme activity when combined with other processes, such as high pressure and/or heat, contrary to the minimal inhibitory effects of individual application (Mason & Paniwnyk, 2003; Morris et al., 2007).

Although a lot of studies on ascorbic acid or ultrasound have been conducted, the combined treatment with ultrasound and ascorbic acid has not been studied yet. Furthermore, the effects of combined treatment on the changes of monophenolase and diphenolase activities in fresh-cut fruits and vegetables have never been studied. In our previous work, the effects of ultrasound application with ascorbic acid on fresh-cut apples were investigated and a positive effect was observed on the inhibition of discolouration and PPO activity as compared to that of the samples treated separately with ultrasound or ascorbic acid (Jang, Kim, & Moon, 2009).

In the present study, as a continuation of enzymatic browning studies in fresh-cut apples treated by ultrasound combined with ascorbic acid, the characterisation of concentrated PPO enzyme and monophenolase and diphenolase activities in terms of substrate specificities, and changes in peroxidase activity in treated fresh-cut apples were investigated.

2. Materials and methods

2.1. Apples and chemicals

The apples (*Malus domestica* Borkh. cv. Fuji) were harvested in a farm located at Kyungpook National University, Republic of Korea in November 2008 and kept at 4 °C until used. L-Tyrosine, 3-(4-hydroxyphenyl)propionic acid (PHPPA), 3,4-dihydroxy-L-phenylalanine (L-DOPA), 4-methylcatechol, catechol, 3-(3,4-dihydroxyphenyl)propionic acid (DHPPA), and chlorogenic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, Mo., USA) and all the other chemicals, including 3-methyl-2-benzothiazolinone hydrazone (MBTH), *N,N′*-dimethylformamide (DMF), and ascorbic acid, were of analytical grade. The 1% ascorbic acid solutions were cooled to 10 °C before use.

2.2. Sample preparation

The apple discs (5 mm thick, 15 mm diameter), without core and peel, were prepared using a cork borer and a sharp knife. After preparation, the apple discs were randomly divided and immediately applied with each treatment. The control sample (Cont) was rinsed with distilled water and the other discs were treated with either ultrasound (US), 1% ascorbic acid (AA), or a combination of ultrasound and 1% ascorbic acid (UA). An ultrasonic generator with a frequency of 40 kHz (Daihan Scientific Co., Ltd., Republic of Korea) was used for the ultrasound treatment. Fifteen discs were packed in polypropylene bags (10 × 10 cm, 0.04 mm thick) and sealed after draining. The samples were stored at 10 °C and analysed after 0 (treatment day), 4, 8, and 12 days of storage.

2.3. Enzyme extraction

The apple discs were homogenised in a twofold amount of chilled 50 mM sodium phosphate buffer (pH 5.0 for crude enzyme extraction and pH 7.0 for partial purification) containing polyvinylpolypyrrolidone (50 g/l) for 2 min using a homogenizer. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 16,000×*g* for 30 min at 4 °C. The supernatant solution was used in experiments.

2.4. Protein determination

The protein content in each enzyme was quantified using QuantiPro™ BCA Assay Kit (Sigma–Aldrich Chemical Co., St. Louis, Mo, USA). Bovine serum albumin was used as standard (Mdluli, 2005). Absorbance at 520 nm was determined using a plate reader (Victor 3, Perkin Elmer, USA).

2.5. Assay of enzyme activity

The monophenolase and diphenolase activities of PPO enzyme from crude enzyme were assayed by measuring the increase in absorbance at 494 nm for 4-methylcatechol, 505 nm for DHPPA, and 500 nm for all other substrates (Espín, Morales, Varón, Tudela, & García-Cánovas, 1995; Winder & Harris, 1991). The reaction mixture contained assay buffer (4% DMF), 5 mM of substrate solution, and 20.7 mM of MBTH and this gave final concentrations of 50 mM sodium phosphate buffer, 2% DMF, 1 mM substrate, and 6 mM MBTH, and a final pH of 5.0. The reaction mixture was incubated at 37 °C for 10 min. The crude enzyme extract was then added and the cuvette contents were mixed by inversion. The absorbance was measured using a spectrophotometer after additional incubation at 37 °C for 5 min. The enzyme activity was expressed as units of enzyme/mg protein and one unit was defined as an increase in absorbance of 0.001.

The peroxidase activity of the crude enzyme was also determined spectrophotometrically using the modified method of Aydin and Kadioglu (2001). The assay mixture contained 20 mM guaiacol, an equal volume of 40 mM hydrogen peroxide, and 50 mM sodium phosphate buffer (pH 5). Changes in the absorbance at 475 nm were monitored for 3 min using a spectrophotometer after the crude enzyme solution was added. One unit was defined as an increase in absorbance of 0.001 per min and the enzyme activity was expressed as units of enzyme/mg protein.

2.6. Characteristics of the concentrated polyphenol oxidase

2.6.1. Enzyme concentration and activity determination

Prepared supernatants from the treated apple discs were collected through centrifugation. Briefly, the supernatants were placed in ultrafiltration tubes (Vivaspin 20, Sartorius, Germany) and centrifuged at 1500×*g* at 4 °C to remove proteins with molecular weights of less than 30 kDa. Sodium phosphate buffer (50 mM, pH 7) was added to the supernatant in equivalent volume and centrifugation was repeated to obtain a final volume of 2.5 ml. After protein determination, a concentration adjustment was performed.

The reaction mixture for measuring PPO activity included 20 μl of the concentrated enzyme and 100 μl of 10 mM catechol solution in 50 mM sodium phosphate buffer (pH 5). Absorbance at 405 nm was measured using a plate reader. One unit of enzyme activity was defined as an increase in absorbance of 0.001 per min and the enzyme activity was expressed as units of enzyme/mg protein.

2.6.2. Semi-native polyacrylamide gel electrophoresis

Electrophoresis was conducted on a Mini-Protean® three Cell electrophoresis unit (Bio-Rad, USA) equipped with a power supply (SP-250, Seoulin Scientific Co., Ltd. Republic of Korea). Eight per cent polyacrylamide gels were used and sample buffer with a 2% sodium dodecyl sulphate (SDS) was added to each enzyme concentrate without heating (Voulhoux, Bos, Geurtsen, Mols, & Tommassen, 2003). After electrophoresis, the gels were sufficiently rinsed with sodium phosphate buffer solution in order to remove the remaining SDS. One gel was stained with Coomassie Blue R-250 to determine the PPO molecular weight and the other gel was treated with a catechol paper to determine the PPO activity. The catechol paper was prepared by immersing it into a 50 mM catechol

solution, followed by drying (Cheng, Huang, Pan, Lin, & Mao, 2007), and was used immediately after preparation. The intensity and molecular weight of the developed bands were determined using a Gel-Pro Analyser software (Media Cybernetics, Silver Spring, USA).

2.7. Statistical analysis

Analysis of Variance and Duncan's multiple range tests were performed using the SAS programme version 9.1 for windows. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Substrate specificity of crude apple PPO

L-Tyrosine and PHPPA as monophenolic substrates, and L-DOPA, 4-methylcatechol, catechol, and DHPPA as diphenolic substrates were examined and their activities were compared with the activity in the presence of DHPPA as 100%, respectively (Table 1). All of the substrates were oxidised considerably by 'Fuji' apple PPO and the diphenolase activities were relatively higher than monophenolase activities. The crude PPO showed the highest activity towards PHPPA and DHPPA as monophenolic and diphenolic substrates, respectively. The enzyme showed the lowest activity towards L-tyrosine, which is in accordance with the previous reports for PPO enzymes from other plant sources (Kolcuoğlu, Colak, Sesli, Yildirim, & Saglam, 2007; Özen, Colak, Dincer, & Güner, 2004).

3.2. Effect of ultrasound and ascorbic acid on monophenolase activities

The monophenolase activities of the treated fresh-cut apples are presented in Fig. 1. Since the monophenolase activity of PPO is generally considered as the first step in enzymatic browning because the enzyme catalyses the hydroxylation of monophenols to *o*-diphenols (Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995), a lower activity can be interpreted as inhibition of enzymatic browning. In monophenolase activity, different trends were observed following various substrates application. When L-tyrosine was used as substrate, the activity in AA-treated samples increased dramatically at the end of storage period. Higher concentrations of ascorbic acid can temporarily retard the induction period, however, monophenolase activity increases rapidly when all the ascorbic acid has been consumed (Ramon, Neptuno, & Francisco, 1993). Lower activities in UA-treated samples were initially observed regardless of the substrates, although the activity on L-tyrosine steadily increased during storage. The significantly lowest activity in UA-treated samples using PHPPA as substrate was maintained. McHedlishvili et al. (2005) reported that monophenolase lost half of its activity in PPO from tea leaf after 2 h incubation at 30 °C and the monophenolase activity is more thermolabile as compared with the diphenolase activity. In contrast, our results showed the inhibition rate of monophenolase activity by UA treatment ranged from 75% to 90%. Therefore, UA treatment effectively inhibited monophenolase activity, unlike in the individual application of ultrasound or ascorbic acid.

Table 1
Substrate specificities of 'Fuji' apple polyphenol oxidase.

Substrate	Wavelength (nm)	Relative activity (%)[a]
Monophenols		
L-Tyrosine	500	1.3 ± 0.2
PHPPA	500	6.4 ± 0.1
Diphenols		
L-DOPA	500	11.3 ± 0.5
4-Methylcatechol	494	90.1 ± 1.8
Catechol	500	28.6 ± 1.2
DHPPA	505	100 ± 0.9
		168.9 ± 1.5[b]
Chlorogenic acid	500	37.5 ± 1.1

[a] Assay medium: 50 mM sodium phosphate buffer (pH 5), 2% DMF, 1 mM substrate, and 6 mM MBTH at 37 °C.
[b] Specific activity (U/mg) using DHPPA as substrate.

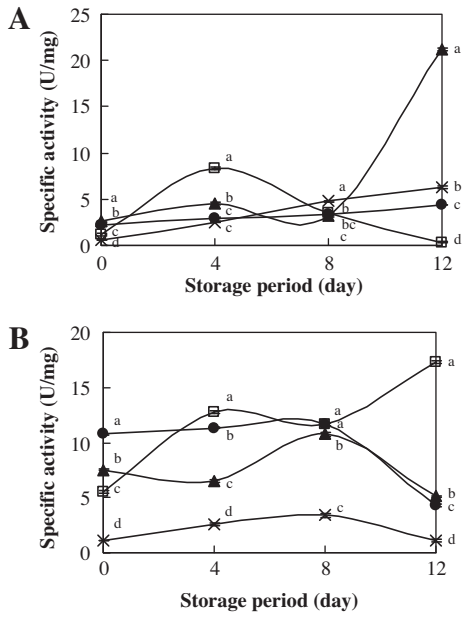

Fig. 1. Monophenolase activity of fresh-cut 'Fuji' apples by L-tyrosine (A) or PHPPA (B) as substrate. (●) control (Cont); (□) ultrasound alone (US); (▲) ascorbic acid alone (AA); (×) ultrasound and ascorbic acid (UA) as treatments. Means ($n = 3$) with different letters are significantly different at 5% level.

3.3. Effect of ultrasound and ascorbic acid on diphenolase activities

The changes in diphenolase activities in the treated fresh-cut apples during storage are illustrated in Fig. 2. The diphenolase activities, measured using several substrates, showed a similar pattern of change during storage. The significantly highest diphenolase activities were observed in Cont and US-treated samples. Especially, US-treated samples showed significantly higher activities when L-DOPA and 4-methylcatechol were used as substrates than Cont during storage. Higher activities in US-treated samples were observed at the end of storage period as compared to that of other samples. These results indicate that ultrasound treatment stimulates diphenolase activity. They are also in agreement with the results obtained by Wu and Lin (2002), who observed that the PPO activities of *Panax ginseng* cells showed a sharp increase immediately after ultrasonication. Lower values, with an increasing trend, in diphenolase activities were observed in AA-treated samples during storage. From the results, it was found that the remaining ascorbic acid on the surface of fresh-cut apples just after processing acted as a reducing agent and thereafter its reducing ability gradually disappeared towards the end of the storage period.

On the other hand, UA treatment almost completely inhibited the diphenolase activity on the treatment day and also showed the lowest activity during the entire storage period. The diphenolase activities in UA-treated samples using all substrates, except 4-methylcatechol, increased slightly for eight days, followed by a decrease to the initial level. The effectiveness of ultrasound in food preservation can be ameliorated by combination with other

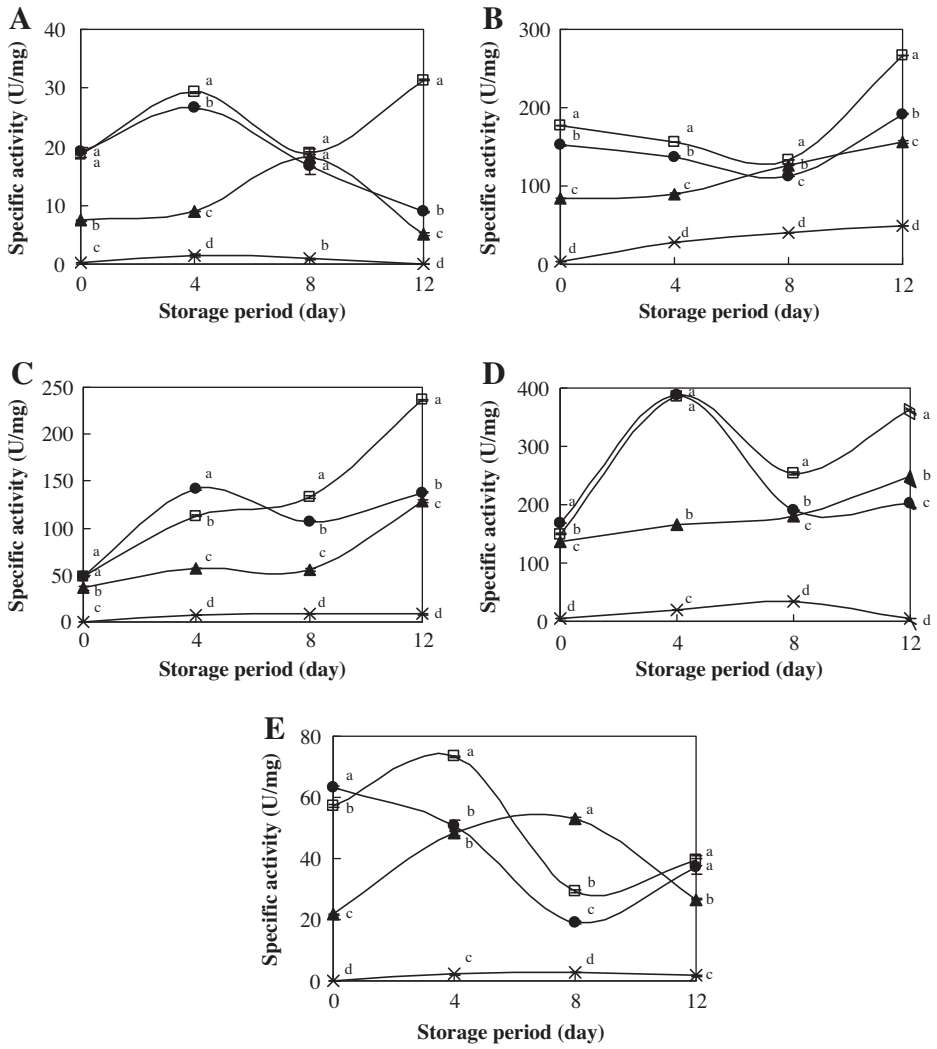

Fig. 2. Diphenolase activity of fresh-cut 'Fuji' apples by L-DOPA (A), 4-methylcatechol (B), catechol (C), DHPPA (D), or chlorogenic acid (E) as substrate. (●) Control (Cont); (□) ultrasound alone (US); (▲) ascorbic acid alone (AA); (×) ultrasound and ascorbic acid (UA) as treatments. Means (n = 3) with different letters are significantly different at 5% level.

treatments, such as heat and/or high pressure, since the resistance of microorganisms and enzymes to ultrasound is very high. To attain the desired levels of effectiveness, it may require prolonged ultrasonication for several hours, however, severe changes could occur in the fresh fruits and vegetables (López et al.,1994; Mason & Paniwnyk, 2003). In this study, it was found that simultaneous treatment with ultrasound and ascorbic acid is capable of effectively inhibiting diphenolase activities even with a relatively short treatment of time without severe appearance deterioration in comparison with thermal treatment (McHedlishvili et al., 2005). Asemota, Wellington, Odutuga, and Ahmad (1992) reported that diphenolase activities in cut yam increased steadily up to the third week of storage and the browning intensity and rate after cutting were very closely correlated with the diphenolase activities (Wu & Lin, 2002). Therefore, the UA-treated samples were able to maintain their lighter colour (Jang et al., 2009) due to the fact that the UA treatment directly affects diphenolase activity and its inhibitory effect against the enzyme was maintained in fresh-cut apples during storage.

3.4. Effect of ultrasound and ascorbic acid on peroxidase

Although it has been recognised that PPO is the main enzyme related to enzymatic browning on fresh-cut apples, it is also necessary to study the changes in POD enzymes as they can also contribute to the discolouration in fresh-cut products. Changes in POD activity in fresh-cut apples were clearly distinguished by the different treatments (Fig. 3). The POD activity in Cont fluctuated with the highest value on the forth day of storage. On the other hand, in US-treated samples significantly low POD activity was detected, when compared to Cont and AA-treated samples at treatment day. However, the POD activity of US-treated samples showed steady increase with time. There have been conflicting reports on the inhibitory effect on POD enzyme by ultrasound. Cruz, Vieira, and Silva (2006) reported that the increase in POD activity in watercress with ultrasound was observed at low temperatures and Wu and Lin (2002) also found that the POD activity was enhanced with the increase in ultrasound intensity. On the other hand, Mason, Paniwnyk, and Lorimer (1996) reported that the original activity of commercially purified POD was progressively reduced by 90% with ultrasound application for over 3 h. The AA-treated sample showed high activity immediately after processing, but the activity decreased until eighth day of storage. With the exception of the high POD activity on treatment day, our results are consistent with those of Lamikanra and Watson (2001), who reported that the presence of ascorbic acid effectively reduced the POD activity in the fresh-cut cantaloupe melon. The researchers also indicated that

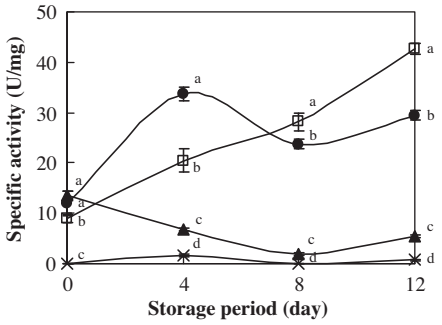

Fig. 3. Peroxidase activity of fresh-cut 'Fuji' apples. (●) Control (Cont); (□) ultrasound alone (US); (▲) ascorbic acid alone (AA); (×) ultrasound and ascorbic acid (UA) as treatments. Means ($n = 3$) with different letters are significantly different at 5% level.

the reduced POD activity in the fruit treated with ascorbic acid could be the result of a lower oxidative stress on the fruit surface due to the antioxidant ability of ascorbic acid. In the present study, the UA treatment effectively reduced the POD activity in fresh-cut apples during the entire storage period. It is inferred from these results that UA treatment allowed the ascorbic acid to act inside the cells disrupted by ultrasound treatment, hence, it was possible to inhibit the POD activity by enhanced antioxidant action.

3.5. Characteristics of the concentrated PPO

Semi-native electrophoresis was performed to detect any electrophoretic changes or loss of PPO activity in fresh-cut apples treated with ultrasound and ascorbic acid. The approximate molecular weight of the main bands of the concentrated PPO in treated apples was calculated to be 63 kDa (Fig. 4A). Similar observations have also been reported in 'Fuji' apple's PPO (Haruta et al., 1998; Murata, Tsurutani, Tomita, Homma, & Kaneko, 1995). The electrophoretic mobility of proteins in concentrated enzymes was little affected by the ultrasound and/or ascorbic acid treatment. When the gel was stained to detect PPO activity, there was a decrease in the intensity by AA treatment (Fig. 4B), which is in agreement with the results obtained by Golan-Goldhirsh and Whitaker (1984). On the other hand, the PPO activity was slightly enhanced by US treatment. Differences in the band intensity closely resembled the enzyme activities that were directly measured (Fig. 4C). The indistinct protein band was observed in lane four from UA-treated samples (Fig. 4B). This means that simultaneous treatment with ultrasound and ascorbic acid has synergistic inhibitory effects on PPO activity without deformation of enzyme protein, whilst ascorbic acid behaves as reversible and temporary PPO inhibitor and ultrasound treatment has no effect on enzyme inhibition.

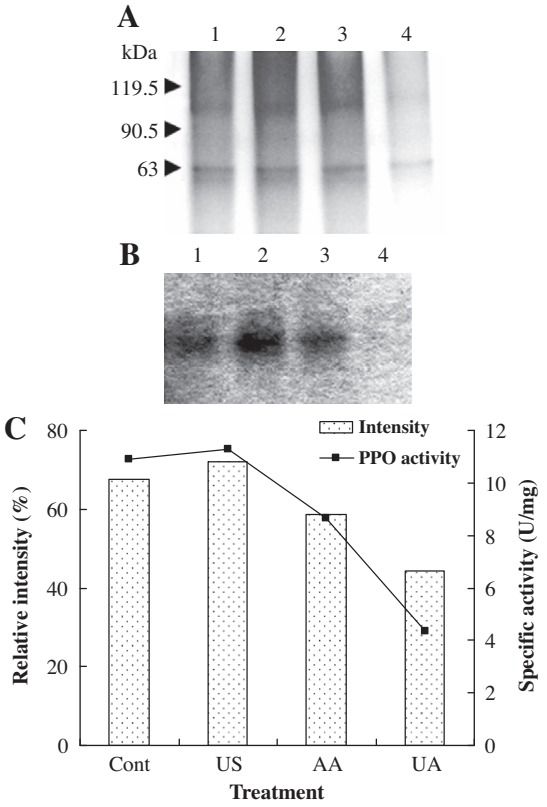

Fig. 4. Electrophoretogram and intensity of semi-native electrophoresis of PPO from apple treated by ultrasound and ascorbic acid. After electrophoresis, each part of the gel was stained for protein with Coomassie Blue B-250 (A) and for activity with catechol paper (B). The intensities of the bands stained with a catechol paper were compared with the measured PPO activities (C). Treatments according to lanes: 1, control (Cont); 2, ultrasound (US); 3, ascorbic acid (AA); 4, ultrasound and ascorbic acid (UA).

4. Conclusion

This study demonstrated that several enzymes related to browning of fresh-cut 'Fuji' apples were inhibited by the combined treatment with ultrasound and ascorbic acid during storage. This treatment was considerably effective in deactivating PPO and POD, whilst the individual application of ultrasound or ascorbic acid did not inactivate the enzymes. The inhibitory effect of this simultaneous treatment resulted from different mechanisms, rather than from the individual use of ultrasound or ascorbic acid, as manifested by the stronger inhibitory effect on the diphenolase activity compared to that of the monophenolase activity, and the loss of PPO activity without electrophoretic migration of the protein. Therefore, results of this study suggest that combined treatment with ultrasound and ascorbic acid has noteworthy possibilities in improving the qualities of fresh-cut fruits and vegetables. Further research is needed for the establishment of optimum treatment conditions in order to completely inactivate the enzyme activities.

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